

**REMARKS**

Claims 1-29 are pending in this patent application. By this amendment, the specification is amended to include sequence listing identification numbers to match those in the sequence listing filed on February 10, 2003. No new matter has been added. Early and favorable action on the merits are respectfully requested.

Respectfully submitted,

PILLSBURY WINTHROP LLP

By: 

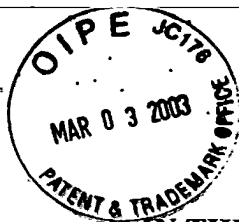
Robin L. Teskin  
Reg. No. 35,030

RLT:af

1600 Tysons Blvd  
McLean, VA 22102  
(703) 905-2200 2085  
(703) 905-2500 Facsimile

Attachment:  
Appendix

*Thomas C. Cady*



## APPENDIX

### IN THE SPECIFICATION:

**Marked up version of the paragraph starting at page 8, lines 4-11 is below:**

In another preferred embodiment, the step of inactivating the catalytic activity of one subunit of the restriction endonuclease comprises identifying the catalytic domain of the subunit and subsequently introducing mutations into the catalytic domain by site-specific mutagenesis. In this embodiment the catalytic domain may be identified by any suitable method, preferably by comparing the protein sequence of the subunit with the protein sequence motifs from other restriction endonucleases. By "protein sequence motifs" it is meant in particular the sequences of the known catalytic domains of other restriction endonucleases, such as the (E/D) $X_{9-15}$ EXK (SEQ ID NO: 9) motif characteristic of some endonucleases.

**Marked up version of the paragraph starting at page 16, line 7 is below:**

FIGURE 1 shows the amino acid sequence of restriction endonuclease *Bpu10I*  $\alpha$  (SEQ ID NO: 1) and  $\beta$  (SEQ ID NO: 2) subunits.

**Marked up version of the paragraph starting at page 16, lines 24-25 is below:**

FIGURE 8 (SEQ ID NOS 3-5, respectively, in order of appearance) shows a scheme for vector preparation for ligase independent cloning method using site specific nickases.

**Marked up version of the paragraph starting at page 16, lines 27-28 is below:**

FIGURE 9 (SEQ ID NOS 6-8, respectively, in order of appearance) shows a scheme of preparation of covalently closed DNA molecules using site specific DNA nickases.

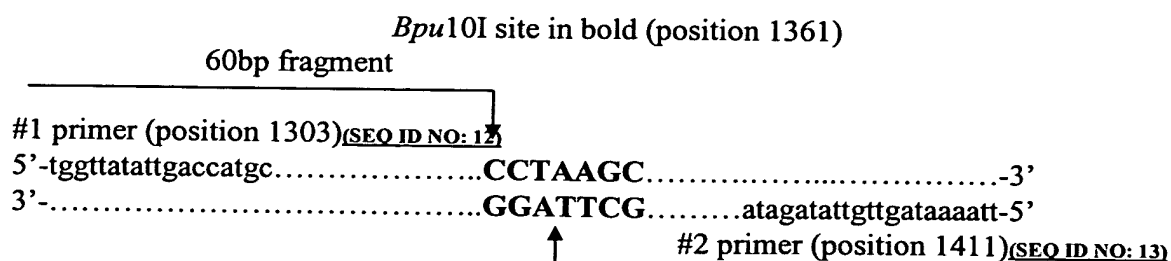
**Marked up version of the paragraph starting at page 17, lines 15-25 is below:**

In order to confirm this proposition point mutations inactivating catalytic activity but retaining protein-protein interaction were introduced into  $\alpha$  and  $\beta$  subunits of *Bpu10I* Enase. For this purpose amino acid sequences of both subunits were analysed trying to identify catalytic centres analogous to those found in other RE, e.g. motif (E/D) $X_{9-15}$ EXK (SEQ ID NO: 9) characteristic for some RE, and the SD<sup>171</sup>X<sub>8</sub>E<sup>180</sup>XK<sup>182</sup> (SEQ ID NO: 10) motif in the N-terminal domain of *Bpu10I*  $\alpha$  and TE<sup>168</sup>X<sub>8</sub>E<sup>177</sup>XK<sup>179</sup> (SEQ ID NO: 11) motif in the N-terminus of  $\beta$  subunit that corresponded quite well to the consensus one were identified. On the basis of these observations the presumption may be made that (E/D) $X_8$ EXK (SEQ ID

**NO: 18)** motifs might be the catalytic/magnesium binding centres of the proteins described therein. This region was chosen as the target for point mutagenesis and series of mutations were introduced by standard PCR techniques both into  $\alpha$  and  $\beta$  subunits (Fig. 1, in which the positions where amino acid substitutions were introduced are underlined.).

**Marked up version of the paragraph starting at page 18, line 8 to page 19, line 16 is below:**

E180Q mutant of  $\alpha$  subunit and E177A mutant of  $\beta$  subunit were chosen for further experiments. Proteins were purified to near homogeneity (see Example 1) and it was shown by several different methods (see Example 2, 3) that when E180Q mutant of  $\alpha$  subunit ( $\alpha$ E180Q) and native  $\beta$  subunit are combined in the reaction mixture the only one strand with the sequence 5'-CC<sup>^</sup>TNAGC-3' is effectively nicked. *Vice versa*, the presence of native  $\alpha$  subunit and E177A mutant of  $\beta$  subunit ( $\beta$ E177A) in the reaction mixture results in the specific nicking of the opposite DNA strand with the recognition sequence 5'-GC<sup>^</sup>TNAGG-3' (Fig. 3). The experiment was performed as follows:  $\Phi$ X174 plasmid DNA, a set of specific primers (#1:5'TGGTTATATTGACCATGCG3' (SEQ ID NO: 12), position 1303; #2:5'TTAAAATAGTTGTTATAGATA3' (SEQ ID NO: 13), position 1411), dNTPs and  $\alpha$ [<sup>32</sup>P]dATP were used in the extension reaction with T7 DNA polymerase through unique *Bpu*10I recognition site (position 1361). After inactivation of polymerase by heating at 65°C for 15 min. labelled extension products were digested in parallel with *Bpu*10I restriction endonuclease, N.*Bpu*10I $\alpha$ ( $\alpha$ + $\beta$ E177A) and N.*Bpu*10I $\beta$ ( $\alpha$ E180Q+ $\beta$ ). Digestion reactions were analysed by 10% denaturing PAGE. Due to different distance from the *Bpu*10I recognition site to the primer annealing sites (60 bp and 46 bp) on the top and bottom DNA strands the cleaved strand could be specifically identified on the gel. In Figure 3, lane 1 shows labelled  $\Phi$ X174 DNA digested with *Bpu*10I restriction endonuclease, lane 2 shows labelled  $\Phi$ X174 DNA digested with N.*Bpu*10I $\alpha$  ( $\alpha$ + $\beta$ E177A), and lane 3 shows labelled  $\Phi$ X174 DNA digested with N.*Bpu*10I $\beta$  ( $\alpha$ E180Q+ $\beta$ ). Primers and fragments cleaved with each nickase are shown below:



---

#### 46bp fragment

Thereby obtained data confirm the hypothesis that a restriction endonuclease recognizing a non-palindromic DNA sequence can be converted by site - specific (or non specific) mutagenesis into a specific nickase cleaving only one DNA strand. These data also confirm the presumption that in the native R.*Bpu10I* enzyme, the  $\alpha$  subunit is responsible for cleavage of DNA strand with the sequence 5'-GC<sup>^</sup>TNAGG-3', while the  $\beta$  subunit - with the sequence 5'-CC<sup>^</sup>TNAGC-3'.

**Marked up version of the paragraph starting at page 27, line 14 to page 28, line 8 is below:**

#### *Example 4*

#### *Vector preparation for ligation independent cloning method.*

pUC57-PKA2 vector plasmid possessing *PstI* recognition site flanked by two inverted *Bpu10I* recognition sites was constructed by ligating the following cassette into *PaeI-KpnI* digested pUC57 (Fermentas):

*PstI* *Bpu10I* $\alpha$

5' - CCTAAGCTCACTCTCAATGGTCTGCAGAGGTCAGACACGCTTAGGCATG-3' (SEQ ID NO: 14)

3' - CATGGGATTTCGAGTGAGAGTTACCAGACGCTCTCCAGTCTGTGCGAATCC-5' (SEQ ID NO: 15)

*Bpu10I* $\alpha$

20  $\mu$ g of plasmid pUC57-PKA2 were treated with N.*Bpu10I* $\alpha$  ( $\alpha$ + $\beta$ E177A) cleaving 5'-GC<sup>^</sup>TNAGG-3' prepared as described in Example 1 in *Bpu10I* buffer in 400  $\mu$ l total reaction volume at 37°C for 3 hours. Nicking enzyme concentration in the reaction mixture was 2pmol/ $\mu$ l. After nicking reaction was complete as confirmed by agarose gel electrophoresis 10 units of *PstI* restriction endonuclease were added into reaction mixture and incubation was prolonged at 37°C for another 2 hours. The reaction was terminated by phenol/chloroform deproteinisation followed by DNA precipitation with ethanol. Digested vector DNA was then incubated with 500 u of *E.coli* Exonuclease III in Exo III buffer (Fermentas) at 25°C for 1 min. Exonuclease degradation should result in the formation of the vector molecule possessing 18 nt and 21 nt single stranded protruding 3' ends.

**Marked up version of the paragraph starting at page 28, lines 10-14 is below:**

Following uridinilated primers possessing 5' ends complementary to vector DNA sequence and their 3' ends complementary to phage lambda sequence (EMBL/GenBank Acc. No. J02459, 2761-3678) have been synthesized:

Seq #1: 5'-CGUGUCUGACCUGAAAAAATA-3' (**SEQ ID NO: 16**)

Seq #2: 5'-GCUCACUCUCAAUGGTGGCGG-3' (**SEQ ID NO: 17**)